THE UNIVERSITY OF ALBERTA

Isolation of a Hypoxic Sub-Population of Bone Marrow Stem Cells

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF MEDICINE

EDMONTON, ALBERTA

FALL, 1981

DEDICATION

For my husband, Bob.
His patience, understanding and
support were invaluable.

Abstract

A modified, collagenase dependent, cell separation technique to isolate hematopoietic stem cells from three anatomic regions of the mouse femur has been defined. Cells recoverable from the central marrow cavity and endosteal regions were morphologically similar to unseparated bone marrow cells, and produced large, speading colonies in assays for committed granulocyte-macrophage progenitor cells (CFUc). Cells isolated from the compact bone were morphologically similar to those recovered from other regions, but produced smaller, exclusively compact colonies in CFUc assays. This unique pattern of CFUc growth was independent of genetic strain and gender differences. However, the number of CFUc that could be harvested from compact bone cells was significantly lower in mice of 6 months of age than mice of 6-12 weeks of age.

Compact bone-derived CFUc exhibited traits usually associated with hypoxic cells. They were sensitive *in vivo* to the cytotoxic effects of the hypoxic cell sensitizer, misonidazole. They were also relatively radioresistant when compared to the radiation sensitivities of CFUc isolated from other regions of the femur. Such radioresistance is taken as indirect evidence of hypoxia. Experiments which demonstrated that CFUc can survive brief periods of hypoxia *in vitro* supported the possibility that these cells may be hypoxic *in vivo*.

The cells in the compact bone may play a role in the regeneration of active hematopoiesis following injury to the marrow. Animals which were pre-treated with misonidazole, to which compact bone CFUc are sensitive, had a reduced ability to survive challenge with irradiation or cyclophosphamide. In these studies, deaths were attributable to hematopoietic failure.

Acknowledgements

I wish to thank Mr. Bert Meeker and Dr. John Pedersen for their assistance with the animal survival studies.

I would also like to thank Dr. Andrew Belch for many helpful discussions.

A special thanks is extended to Dr. J. Donald Chapman who gave freely of his time and expertise throughout the course of this work. His enthusiasm for learning has made my association with him a most exciting one.

These studies were supported in part by a grant from the National Cancer Institute of Canada.

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INTRODUCTION

It has been assumed that bone marrow stem cells exist in a uniformly well-oxygenated environment. This belief arises from studies of the irradiation response of hematopoietic stem cells (1-3). Exponential survival curves of marrow colony forming units indicated that the bone marrow contains a homogeneous population of cells. In addition, the relatively low D_o doses and significant oxygen enhancement ratios observed in these irradiation studies have suggested that the marrow is a well-oxygenated tissue. The studies described in this thesis report the identification and partial characterization of a sub-population of hematopoietic stem cells, with characteristics similar to those of hypoxic cells. This sub-population of cells is sensitive to the cytotoxic action of the hypoxic cell sensitizer, misonidazole. These cells are also relatively resistant to the effects of ionizing irradiation. In addition, they exhibit a unique pattern of growth in assays of committed granulocyte-macrophage progenitor cells (CFUc), forming smaller, uniformly compact colonies.

Misonidazole is an efficient hypoxic cell radiosensitizing agent (4-10). In both *in vitro* cell cultures and *in vivo* tumor models, misonidazole enhances the response of hypoxic cells to the effects of irradiation. Misonidazole concentrations of 5 mM produce a radiation sensitivity of hypoxic cells which approaches that of oxygenated cells. In addition to its radiosensitizing activity, misonidazole is also selectively cytotoxic for hypoxic cells (11-17). Exposure to 1 - 2 mM of misonidazole for a few hours results in significant cell toxicity for hypoxic cells, with well-oxygenated cells being spared this effect. Earlier studies which investigated the effects of misonidazole on the

murine bone marrow utilized similar concentrations and exposure times (18). It has been assumed that the bone marrow would be spared the cytotoxic and radiosensitizing effects of misonidazole, because the bone marrow is considered to be a well-oxygenated tissue.

Initial studies with mouse marrow, using conventional assay techniques, did not detect any radiosensitization or cytotoxicity of misonidazole on colony forming units (18). Extensive evaluation of pluripotential stem cells (CFUs) and CFUc in normal and tumor bearing mice did not reveal any evidence of misonidazole-associated damage. In contrast, studies of bone marrow CFUc obtained from patients receiving misonidazole have demonstrated that hematopoietic toxicity is associated with its use (19). This cytotoxicity may be interpreted as evidence for a sub-population of human hematopoietic stem cells that is relatively hypoxic.

The variant results obtained from studies of misonidazole's effect on human and murine bone marrow have led to a re-evaluation of the animal data. The specific aim of this thesis was to determine if there is any evidence which indicates that a sub-population of mouse bone marrow cells is hypoxic.

The possibility that rodent bone marrow may contain several distinct microenvironments is based on the observation that hematopoietic stem cells are not uniformly distributed in the femur (20-24). Three populations of stem cells can be separated according to their spatial distribution within the femur (25). Most of the stem cells are located in the central marrow cavity which runs parallel to the longitudinal axis of the femur. Stem cells are also found in close association with the endosteum which encircles the cavity. A third

population of stem cells resides in the compact bone. The techniques which are routinely used to collect cells for culture retrieve only those cells which are easily flushed from the marrow cavity. The residual populations are seldom analyzed. In these studies, each population was examined separately for evidence of hypoxia.

Various methods have been used to accomplish the separation of marrow cells according to their spatial distribution. The most commonly employed techniques utilize either physical separation of the bone, or enzymatic digestion of the bone (20-25). Chapter One of this thesis details the techniques which were developed in order to achieve accurate and reproducible separations. A description of each sub-population of hematopoietic stem cells is provided. This description includes gross morphological characterization as well as an analysis of each population's response in tissue culture.

Chapter Two of this thesis examines the effects of misonidazole, irradiation and hypoxia on the stem cell populations. These probes can provide evidence that a given cell population is hypoxic. Cytotoxicity of misonidazole to any cell population is taken as indirect evidence for hypoxia. When cells are hypoxic, they are also relatively resistant to the effects of ionizing irradiation (26). These studies demonstrate that the stem cells which are located in the compact bone are more sensitive to misonidazole cytotoxicity and are more resistant to the effects of ionizing radiation than are the stem cells in the central region. Chapter Two also examines the effects of short periods of hypoxia on stem cells. If one theorizes that some stem cells exist in a relatively hypoxic environment, then one should be able to demonstrate *in vitro* that hypoxic conditions are not incompatible with continued

cell survival. Thus a description of the response of stem cells to hypoxia is provided.

Chapter Three of this thesis presents data from studies which investigated the effect of misonidazole pre-treatment on the survival of animals following whole body irradiation or cyclophosphamide administration. Such studies can provide indications of the possible role of the fraction of hypoxic stem cells in hematopoietic recovery. If this fraction serves as a reserve of stem cells, then damage to this population by misonidazole may impair the ability of the host to recover from challenges by known myelotoxic agents.

MATERIALS AND METHODS

Animals

Animals used in these studies were obtained from breeding stock maintained at the University of Alberta Health Science animal facilities. Mice were housed five per cage with rodent laboratory chow (Ralston Purina Co.) and water available ad.lib. Mice of the following strains were studied: C_{57}Bl/10J, Balb/C and BDF_1. Except in studies that investigated the effect of age on the cellular content of the femur, all animals were between six and nine weeks of age at the time of experimentation. Only male mice were used in these studies, except for studies that investigated gender-associated differences in the cellular content of femurs.

Media

Saline solutions (0.9%) were prepared from reagent grade NaCl (Fisher Scientific Co.) and distilled water. Saline solutions were filtered before use with a 110 ml capacity Nalge filter unit containing a 0.45 μm filter (Nalge Co.) Minimal essential media (MEM) used in these experiments was purchased from GIBCO. Hanks balanced salt solution (HBSS) was prepared from powdered material with sterile distilled water and reagent grade $NaHCO_3$ (Fisher Scientific Co.) according to the manufacturer's instructions. MEM Alpha was prepared similarly with sterile distilled water and $NaHCO_3$. Both 1X and 2X concentrations were prepared. No additional nutrients were added to either media, nor were any antibiotics used. Prepared media was filtered with a Millipore all- glass filter apparatus with a 0.45 μm filter in place (Millipore Corp.) Media was stored at $4^{\circ}C$ prior to use. Storage time did not exceed three weeks.

L-cell conditioned media was used as a source of colony stimulating activity. Mouse fibroblasts (L-cells) (Flow Laboratories) were grown as a monolayer culture. Twenty thousand cells were added per one ml of media (1X MEM Alpha with 15% fetal calf serum). Approximately 100 ml cells in media were cultured in a 500 ml capacity sterile glass bottle. After seven days incubation at 37°C in a humidified atmosphere of 7.5% CO_2 in air, the supernatant was decanted, filtered with a Nalge filter unit and stored at -20°C until used. Fetal calf serum used in these studies was purchased from Flow Laboratories and stored at -20°C prior to use. Horse serum was purchased from GIBCO and stored at -20°C. Two percent MEM Alpha methylcellulose solutions were prepared by adding 250 ml boiling distilled water to a sterile flask containing 10 g Methocel MC 4000 cP purum grade (Fluka AG). This material was gently stirred until contents were at room temperature. Two hundred fifty ml of sterile 2X MEM Alpha media was then added, and the mixture was allowed to stir at 4°C until the material had cleared (usually one to two days). The methocel solution was then decanted into 100 ml sterile flasks and held at 4°C until use.

Drugs and Biologicals

Misonidazole (1-(2-nitro-1-imidazole)-3-methoxy-2-propanol) was obtained in cystalline form from Hoffmann-LaRoche. Immediately prior to use, misonidazole was diluted to the appropriate concentration with 0.9% NaCl. Collagenase (Cl. histolyticum) was purchased from GIBCO as a lyophilyzed powder at a concentration of 135 units/mg. Working solutions of 1 mg/ml were prepared by dilution with HBSS immediately prior to use. Cyclophosphamide (200 mg/vial) was obtained in powdered

form from Bristol Labs, and was reconstituted with distilled water immediately prior to use.

Cell Counts

All cell counts were electronically determined with a Coulter Counter model ZBI (Coulter Electronics of Canada Ltd.) Coulter Isoton was used as a diluent and Coulter Zap Isoton was used as a lysing agent. Duplicate counts of each sample were routinely performed, with the numerical average of six individual counts used as the cell count of the sample.

CFUc Culture

Techniques used for the culture of CFUc were a modification of those described by Pluznik and Sachs (27) and Bradley and Metcalf (28). Cells to be plated were suspended in a mixture of 74% MEM Alpha, 15% fetal calf serum, 10% L-cell conditioned media and 1% methylcellulose. Final cell concentrations ranged from 7.5×10^4 to 10^5 cells per ml. Each experimental group was plated in quadruplicate with one ml of culture material per 10 X 35 mm tissue culture dish (Corning Glass Works). All dishes were incubated in a humidified atmosphere of 7.5% CO_2 in air at a constant temperature of $37^{\circ}C$ with a water-jacketed National Incubator, Model 3331 (National Appliance Co.). After seven days of incubation, colonies were scored with the aid of a Diavert light microscope (Wild Leitz Canada) at 40X magnification. Aggregates of 50 cells or more were counted as colonies. The numerical average of four plates per experimental group was determined and the results were expressed as the number of colonies per 10^5 cells plated and/or the number of colonies per femur.

Cellular Morphology

Samples of bone marrow were prepared for morphological examination by centrifugation with a Cytospin (Shandon Southern Products). Slides were routinely stained with certified Wright's Stain (Fisher Scientific Co.) and were allowed to air dry before mounting with Permount mounting media (Fisher Scientific Co.). Cellular differential counts were determined at 1000X magnification using a HM-Lux light microscope (Wild Leitz Canada), with 500 cells counted per slide.

The morphology of CFUc was determined by aspirating individual colonies with a finely pulled Pasteur pipet and smearing the cellular contents onto a glass microscope slide. When dry, the slides were stained and mounted in a manner identical to that used for bone marrow cell preparations. The predominate cell type present in each colony was noted upon microscopic examination with a HM-Lux microscope at 1000X magnification.

Collagenase Fractionation

Separation of bone marrow cells according to their spatial distribution within the femur was performed according to modifications of techniques originally described by Kilgore et al. (25). All fractionation procedures occurred at room temperature. Femurs were excised sterilely from animals immediately after cervical dislocation. Each femur was weighed after removal of adherent tissue. Using a 10 ml syringe and a 23 gauge needle, six ml of HBSS was passed through the femur two times. This procedure effectively removed the bone marrow cells which were in the central core of the femur, and which are referred to as fraction I cells. Horse serum (0.6ml) was added to this cell suspension which was then held on ice as further fractionation of

the femur continued. Fraction II cells, which are thought to reside near the endosteum, were obtained by continuous passage of 6 ml HBSS containing 1 mg/ml collagenase through the length of the femur for 10 minutes. At the end of the second procedure, 0.6 ml of horse serum was added to the cell suspension and fraction II cells were held on ice. In the final fractionation procedure which released the cells which are thought to be in association with the compact bone, the previously washed femur was minced into pieces not greater than 1 mm in length using sterile instruments. The bone fragments were then bathed in 3 ml HBSS containing 1 mg/ml collagenase. The suspension was vigorously mixed at two to three minute intervals for a total incubation time of 15 minutes at room temperature. Horse serum (0.3 ml) was then added to the cell suspension. The bone free supernatant was decanted and held on ice until the time of culture. After these fractionation procedures, the bone marrow cells were counted, plated and prepared for morphological examination as previously described.

Nitrogen Chambers

Experiments that required incubation of cells under hypoxic conditions utilized a series of nitrogen chambers designed by J.D. Chapman et al. (29). Stoppered, side-arm tissue culture flasks were serially connected with plastic tubing. Nitrogen (95%) with CO_2 (5%) was continuously passed through the flasks. The apparatus was maintained in a 37^{o}C water bath fitted with magnetic stirring capabilities. Samples were removed through side ports throughout the course of the experiment without affecting the integrity of the hypoxia.

Statistics

These data were analyzed by parametric methods including Student's t-test and Chi Square analysis. Statistical tables found in Biostatistical Analysis (30) were used to determine the levels of significance.

Conditioned Media Dilution Curve

Bone marrow cells were separated into three fractions as described above. Each fraction was assayed for CFUc content in the usual manner, except that the final concentration of L-Cell conditioned media was varied. In addition to the standard concentration of 10% conditioned media, final concentrations of 1%, 5%, 15% and 20% were also assayed. The mean number of colonies stimulated at each concentration was used to determine a conditioned media dilution curve for each fraction.

Collagenase Cytotoxicity Assay

Fraction I cells were removed by passage of 3 ml HBSS through a femur. After mixing, cells were divided into three tubes (1ml/tube). Additional media with or without collagenase was added to each tube, and the cells were incubated at 20°C as follows:

Tube	Additional Media	Final Concentration of Collagenase	Incubation Time
A	2 ml HBSS	0	30 min.
B	2 ml HBSS	1 mg/ml	15 min.
C	2 ml HBSS	1 mg/ml	30 min.

Incubations were terminated with the addition of 0.3 ml horse serum. Cells were maintained on ice after the addition of the serum. Subsequent cell counts and CFUc assays were done as previously described.

Assay for misonidazole cytotoxicity in vitro

Bone marrow cells were separated according to their spatial distribution as previously described. In additon, fraction I cells were flushed from another femur using 6 ml HBSS with 1 mg/ml collagenase. These additional cells were incubated at room temperature for 30 minutes. The incubation was terminated with the addition of 0.6 ml horse serum and transfer to ice. Four populations of cells were then available for assay: fraction I control, fraction I with collagenase, fraction II and fraction III. Misonidazole was added to the media of replicate cultures of each population of cells. The following final concentrations were used: 250, 500 and 1000 uM misonidazole. Bone marrow cells were then assayed for CFUc as previously described. The results of these experiments compared number of CFUc from treated animals with the number of CFUc from saline controls.

Irradiation Procedures

For CFUc assays, animals were irradiated with a ^{137}Cs source (Atomic Energy of Canada) calibrated to deliver 104 rads/minute. For survival studies, animals were irradiated with a ^{60}Co source (Atomic Energy of Canada) calibrated to deliver 134.8 rads/minute.

Survival Studies

Mice matched for age and weight were injected with misonidazole (MIS) or physiological saline according to the following schedule:

	Group I	Group II
0 hr	MIS (0.2 mg/g)	NaCl (0.01 ml/g)
1 hr	MIS (0.1 mg/g)	NaCl (0.01 ml/g)
2 hr	MIS (0.1 mg/g)	NaCl (0.01 ml/g)
3 hr	MIS (0.1 mg/g)	NaCl (0.01 ml/g)

At 3.5 hours, animals (20/group) received the following doses of irradiation:

Group I	Group II
430 rads	530 rads
480 rads	580 rads
530 rads	630 rads
580 rads	680 rads
630 rads	730 rads

A third treatment group received 530 rads before administration of multiple doses of misonidazole described for Group I.

Animals were housed 2-4 per cage and observed over a 30 day period. Cages were inspected twice a day, and careful note was made of any deaths.

In other survival studies, animals were treated with misonidazole or saline as described above. Twenty to forty animals per group received cyclophosphamide at 3.5 hours, at the following doses: 200, 250, 300, 325, 350, 375, 400, 425, or 450 mg/kg. Animals were housed and observed as in the irradiation studies.

CHAPTER ONE

ISOLATION AND PARTIAL CHARACTERIZATION

OF THREE SUB-POPULATIONS OF STEM CELLS

Chapter One

RESULTS

Characterization of Bone Marrow Fractions

Separation of bone marrow cells with collagenase revealed differences in cell number, CFUc number and CFUc morphology among the different fractions of cells. Table I provides a profile of the characteristics of each fraction of cells. The cleaned, excised femurs from which the bone marrow cells were isolated weighed an average of 44 milligrams. Approximately 4 X 10^7 cells were isolated from each femur. Sixty to seventy percent of these cells were found in fraction I, with approximately 25% and 15% being in fractions II and III, respectively. Fractions I and II are similar with regard to the proportions of the cell types enumerated. Fraction III contained fewer recognizable lymphocytes as compared to the other fractions, but this difference was not statistically significant.

When each fraction was assayed for CFUc content, significant differences were noted in the number of CFUc per 10^5 cells plated. Fraction I contained the highest number of CFUc, with approximately one of every 800 cells giving rise to a colony. Fractions II and III contained fewer CFUc, with ratios of one CFUc per each 2000 and 3000 cells respectively. The size of the colonies produced by these CFUc also differed. The colonies arising from CFUc in fraction I and II ranged in size from 50 cells to in excess of 2000 cells per colony. In contrast, the CFUc from fraction III were consistently smaller, rarely exceeding 1000 cells per colony. Doubling the incubation period of fraction III cells did not result in the formation of larger colonies, suggesting that a difference in proliferation rate was not responsible for the observation of smaller colony size.

Table I

Characteristics of Cells Isolated from

Different Regions of the Femur

	Fraction I	Fraction II	Fraction III
	Total Cell Number Mean ± SEM (range)		
C_{57}Bl/10J (male)	$2.9 \pm 0.8 \times 10^7$ (1.7 - 4.0)	$9.4 \pm 0.5 \times 10^6$ (5.2 - 15.3)	$3.2 \pm 0.3 \times 10^6$ (0.9 - 8.7)
C_{57}Bl/10J (female)	$2.3 \pm 0.2 \times 10^7$ (1.9 - 3.7)	$8.6 \pm 0.8 \times 10^6$ (3.6 - 15.9)	$2.2 \pm 0.3 \times 10^6$ (0.9 - 7.5)
Balb/C	$2.0 \pm 0.8 \times 10^7$ (1.6 - 2.3)	$8.3 \pm 0.6 \times 10^6$ (6.1 - 10.3)	$2.7 \pm 0.6 \times 10^6$ (1.0 - 7.9)
BDF_1	$2.2 \pm 0.2 \times 10^7$ (1.1 - 3.0)	$7.2 \pm 0.7 \times 10^6$ (3.1 - 11.1)	$1.5 \pm 0.6 \times 10^6$ (0.9 - 2.4)
$CFUc/10^5$ cells plated	123 ± 8	48 ± 6	35 ± 6
Average Colony Size (range)	(50 - 2000^+)	(50 - 2000^+)	(50 - 1000)
$CFUc/10^5$ cells plated (for animals 6 months old)	147 ± 6	43 ± 13	4 ± 2
Colony Morphology			
macrophage-like	94%	98%	95%
early myeloid	6%	2%	5%
mature myeloid	0%	0%	occasional neutrophil

Differences in colony type were also noted. The CFUc of fractions I and II produced predominately spreading colonies, with some compact colonies also being observed. However, the colonies produced by fraction III CFUc were almost exclusively compact, with few spreading colonies being noted.

Analysis of individual cells from colonies produced by each fraction demonstrated that most of the colonies were composed of monocyte-macrophages. Colonies of early myeloid cells were occasionally observed in each fraction. Colonies containing mature myeloid elements were seen only in cultures of fraction III cells. However, this was a rare observation.

Three strains of mice were used to determine the characteristics of the bone marrow fractions described above. No differences were noted among the strains with respect to any of the parameters tested. In addition, no differences were noted when male and female mice were compared. However, the age of the animal at the time of assay was one factor which altered the results obtained. The influence of age was noted in fraction III. As the age of the animals increased, the number of CFUc found in this fraction decreased. When animals were approximately six months of age, only a few CFUc from fraction III were observed in culture. Conversely, the number of CFUc isolated in fractions I and II did not vary significantly when 6 week or 6 month old animals were compared.

Chapter One

DISCUSSION

In 1968, Carsten and Bond (31) and Van Dyke (32) independently reported that hematopoietic stem cells could be found near the endosteum and in the compact bone. Since that time, other investigators have described the distribution of hematopoietic stem cells outside the medullary cavity of the rodent femur. In this area of study, it is difficult to compare the work of different investigators. There appears to be no agreement as to which specific areas of the femur constitute unique microenvironments with distinct sub-populations of stem cells. Most workers regard the marrow cells in the central marrow cavity as a distinct population of cells within a distinct microenvironment (21-25). Some workers have isolated the hematopoietic cells near the endosteum as a second population (21, 23). Others have defined a third population, those cells which are within the compact bone (25). Finally, in some studies, the populations near the endosteum and in the bone have been combined and treated as a single population (21, 24).

These differing analyses arise in part from the lack of a standardized, well-accepted technique for the isolation of bone marrow stem cells from different areas of the femur. The methods which were first used for this separation relied on physical techniques for separation of the cells from the femur. The axial cells were expelled from the femur with air pressure, or by flushing media through the shaft. Cells were removed from the endosteal area by scraping a split femur or by continually washing an intact femur. Cells found in the compact bone were usually isolated after the femur had been ground with a mortar and pestle. In 1978, Kilgore et al. (25) established a

technique which relied on the digestive action of the enzyme, collagenase, to disrupt the matrix of the bone and release the hematopoietic cells. The description of this technique, which has been published in abstract form, provides only an outline of the method. This thesis begins with a detailed description of the modifications which have been applied to the collagenase method of separating bone marrow cells. These modifications simplify the technique as much as possible, while ensuring optimal and reproducible cell yields.

The optimized technique for separation of hematopoietic stem cells begins with a cleaned, sterilely excised femur. Only femurs which had not been cracked or split were used. The weight of each femur was noted. Because the number of cells recovered from each femur is a function of femur mass, weighing the femurs before assay allowed for an objective selection of femur masses. In these experiments, femurs less than 30 mg were not used. This weighing procedure was included to monitor the excision and cleaning steps. Since animals were matched for age and weight in each experiment, significant differences in femur weight due to physiological variation were unlikely.

After the femurs were weighed, fraction I cells were removed from the central marrow cavity by passing HBSS through the femur. A 23 gauge needle and 10 ml syringe were chosen for this step. Most routine marrow washes employ a 25 or 26 gauge needle. In this case, a 23 gauge needle was chosen because the cells could be expelled more rapidly, and because the diameter of the needle is such that it fitted snugly into the femur shaft. This snug fit freed the experimenter from the need to secure the femur with forceps during the wash procedure.

In the second step, which isolated cells from the endosteal region, the needle was inserted to a point near the middle of the femur. Cells were flushed out by repeatedly drawing media with collagenase through the femur. After five minutes, the position of the femur was reversed and the other end was washed for five minutes. This second washing step was reduced from the 15 minutes suggested by Kilgore et al. (25) with no reduction in the number of cells harvested per fraction.

The final step isolated the stem cells from the compact bone. Bone fragments obtained by gently snipping the femur were bathed in 3 ml of a collagenase solution. This volume had been reduced from 6 ml in order to increase the final concentration of cells per ml. When the volume was reduced, it was no longer necessary to centrifuge the sample in order to obtain adequate cell concentrations for the CFUc assay. In earlier experiments, this centrifugation resulted in a significant loss of cells from this fraction. The reduction in the volume of collagenase used in this step (from 6 ml to 3 ml) did not reduce the cell yield. This third isolation step was further modified by adding a mechanical disruption to the 15 minute incubation period. At two to three minute intervals, the bone fragments were mixed and crushed with a glass rod. This additional crushing greatly increased cell yields over those obtained with an undisturbed incubation.

The final modification involved the substitution of horse serum for fetal calf serum in the termination of each isolation step. This substitution was prompted by the increasing cost and difficulty in obtaining fetal calf serum. Horse serum, which is relatively inexpensive, gave results which were similar to those obtained with fetal calf serum.

In summary, these modifications served to increase cell yields, reduce assay time and decrease the cost of the procedure. This thesis compares the properties of the cells isolated from different areas of the femur using the modified collagenase technique. This comparison provides evidence to suggest that the hematopoietic stem cells which exist in unique microenvironments express unique characteristics.

The hematopoietic microenvironment provides a home for hematopoietic stem cells "by virtue of containing a stroma that exerts a determining influence on these cells and processes" (33). In the adult mouse, hematopoietic microenvironments are found in the spleen and bone marrow. From studies of the development of colony forming units in irradiated animals, it has been determined that the spleen supports predominately erythropoiesis, while the bone marrow provides support for myelopoiesis (33). The femur is a major site of myelopoiesis in the mouse. The architecture of the femur which provides the milieu in which myelopoiesis occurs has been described in detail (34-38). The following summary adapted from these sources (34-38) describes the microenvironments in which each fraction of bone marrow cells was found. The central marrow cavity from which fraction I cells were isolated is filled with a meshwork of vessels and reticular fibers which support the hematopoietic cells. Fraction II cells are located near the endosteal surface of the bone, which encircles the marrow cavity. Fraction III cells reside within the compact bone of the femur, which is characterized by a system of Haversian canals. Because fraction I cells constitute the bulk of the hematopoietic tissue which is found in the femur, studies of the hematopoietic process have largely been based on the response of these cells in various *in vivo* and *in vitro* assays.

The characteristics of the stem cells which are found in other microenvironments are now compared to those of fraction I cells. As shown in Table I, most of the myeloid cells were isolated in fraction I. Of interest is the fact that zones outside the medullary cavity contained appreciable numbers of hematopoietic cells. This is shown in fraction III, which contained up to 20% of the total number of hematopoietic cells which could be isolated from the femur. In some instances, as many as 8 X 10^6 were isolated in this fraction, as compared to an average of 40 X 10^6 total cells per femur.

Similar kinds of cells were found in different areas of the femur. The presence of similar kinds of cells in different microenvironments suggested that the stem cells which give rise to these mature hematopoietic cells could also be found in these microenvironments. However, it is presently not possible to identify hematopoietic stem cells by morphological appearance. Therefore, the presence of stem cells in these areas must be inferred from the results of <u>in vitro</u> colony forming assays. The results of CFUc assays of the cells obtained from different microenvironments are presented in Table I.

The concentration of CFUc found in different areas decreases as the distance from the longitudinal axis of the central cavity increases. The compact bone contained the fewest number of CFUc, both in terms of absolute numbers of CFUc per femur and in terms of the ratio of CFUc to the number of nucleated cells harvested per area. The CFUc in the endosteal region are midway in both absolute number and ratio. These results are similar to those reported by Lord et al. (39). By using variable pressure to extrude cells from the femur, these investigators were able to demonstrate a peak concentration of CFUc well away from the

surface of the bone. This peak presumably corresponds to the peak of CFUc which was found in fraction I cells described in this thesis. The reduced concentration of CFUc found by Lord et al. in the endosteal region corresponds to the fraction II cells of this thesis. Lord and co-workers did not investigate the CFUc in the compact bone.

Studies by Kilgore et al. (25) more closely parallel those described in this thesis, and also note a decrease in CFUc concentration as distance from the center of the marrow cavity increased. The modified collagenase technique used in this thesis recovered, on average, slightly more CFUc in each fraction than was reported by Kilgore et al. However, the gradient of CFUc concentration across the femur was similar.

Fraction I CFUc produced colonies which ranged in size from 50 cells to in excess of 2000 cells. The colonies were composed of monocyte-macrophages or early myeloid cells, and tended to be spreading in shape. These are the characteristics commonly associated with bone marrow colonies (40). The colonies from fraction II CFUc displayed similar characteristics to those from fraction I. However, the colonies produced by fraction III cells were clearly different. The smaller size and exclusively compact shape distinguished them visually from the colonies found in other fractions. Compact colonies are not usually observed in cultures of murine CFUc (40), but have been noted in cultures of marrow cells of other species. Human bone marrow CFUc can produce tight, compact colonies which invariably contain eosinophils (41). Some neoplastic hematopoietic cells also form tight colonies (40). When the morphology of the compact colonies of fraction III was determined, these colonies were found to contain monocyte-macrophages

and myeloid cells of the type found in fraction I and II colonies. None of the colonies contained eosinophils or abnormal cell types.

The three strains of mice which were tested in this thesis were had similar CFUc profiles. Although slightly more CFUc were noted in the fractions obtained from C_{57}Bl/10J mice, the same relative cell numbers and types of colonies were found in each strain. This is in agreement with the observation of Metcalf that similar levels of CFUc are found in the bone marrow of different strains of mice (40). This observation suggests that variation in genetic strains has little effect on the microenvironment of the femur and its ability to support the development of the hematopoietic stem cells [One exception to this is the Sl/Sl^d mouse in which a microenvironmental defect has been noted (42)].

The profile of the CFUc which were obtained from each fraction was the same for both male and female animals. Based on studies of human CFUc, it has been suggested that males, in general, have a lower concentration of bone marrow CFUc, but that these CFUc proliferate more rapidly (43), possibly due to the effects of androgens (44). The studies in this thesis were not able to demonstrate a gender-associated difference in the concentration of CFUc from any fraction of cells, nor was a gender-associated difference in the number of colonies per 10^5 nucleated cells noted. These differences may not have been observed because the murine CFUc were assayed under conditions of maximum stimulation by L-cell conditioned media. This may not have been the case in the earlier human studies, which used unseparated bone marrow as the target. Furthermore, a CSA dilution curve was not provided for evaluation with the human data, leaving open the possibility that the culture conditions had not been optimized for these experiments.

The final aspect of the CFUc profile to be considered in this chapter is the observation of an ageing phenomenon which is associated with fraction III cells. By six months of age, few CFUc could be isolated from the compact bone of the femur. However, the number of CFUc found in the other fractions remained the same. Croizat et al. (45) reported that the absolute number of CFUc per femur increased with age when animals of 3 and 17 months were compared. Studies in this thesis could not confirm such an increase in CFUc. Because this work compared animals of 2 and 6 months of age, a slight increase in CFUc content may not have been large enough to detect. Croizat et al. make no comment with regard to the CFUc in the compact bone. The observed decrease in the CFUc content of fraction III may represent the first report of a decline with age of a population of CFUc in a specific region of the femur. The possibility that this decrease reflects a true physiological decline in CFUc content cannot be excluded. If the process of bone hardening or bone remodelling alters the microenvironment, it may no longer provide a hospitable milieu for hematopoietic stem cells. Because the exact conditions which constitute an adequate hematopoietic microenvironment remain unknown, it is impossible to determine in what manner the bone turnover process may alter its integrity. Another explanation for this observed decrease in CFUc may be that the CFUc migrate with time from this area into the medullary cavity as a part of the normal hematopoietic process. Such a migration could contribute to the rise in CFUc observed by Croizat et al. However, the contribution of each fraction of cells and each microenvironment to the overall scheme of hematopoiesis has yet to be

determined. Therefore, theories concerning the observed decline in the CFUc content of fraction III cells remain untested.

In conclusion, the material presented in this chapter has suggested that three distinct populations of stem cells can be isolated from the mouse femur according to their spatial distribution within the femur. The cells from each fraction share many of the same characteristics, but those of fraction III can be distinguished by a unique pattern of CFUc growth. The data discussed in Chapter Two expands the description of each fraction of cells and presents additional data which suggests that the CFUc of fraction III respond in a unique manner to the stresses of misonidazole, irradiation and hypoxia.

CHAPTER TWO

Response of Bone Marrow Stem Cells to Misonidazole, Irradiation and Hypoxia

Chapter Two

RESULTS

Collagenase Cytotoxicity Assay

No reduction of CFUc was observed when cultures of control cells and cells which had been exposed *in vitro* to collagenase were compared. In these tests for cytotoxicity, collagenase was used in concentrations and incubation times identical to those used in the bone marrow separation technique. The results of three experimental trials are presented in Table II.

L-Cell Dilution Curve

Each fraction of bone marrow cells was assayed for CFUc growth using a range of concentrations of L-cell conditioned media. Each fraction of cells responded maximally to a final concentration of 10% L-cell conditioned media (Table III). The addition of more conditioned media to the cultures (15% or 20%), did not increase the number of colonies over that obtained with a final concentration of 10%. Reducing the concentration of conditioned media reduced the number of colonies produced by each fraction of cells.

Assay for Misonidazole Cytotoxicity *in vitro*

When bone marrow cells from each fraction were cultured in the presence of misonidazole, there was no significant reduction in the number of CFUc compared to untreated controls. The results of two experimental trials are summarized in Table IV. In addition to demonstrating that *in vitro* misonidazole was not cytotoxic for CFUc, these data also suggested that prior exposure to collagenase did not have any effect on subsequent CFUc growth in the presence of misonidazole. This can be determined by comparison of fraction I control and collagenase groups.

TABLE II

Effect of Pre-Incubation with Collagenase on CFUc Growth

Incubations	Mean CFUc/7.5 X 10^4 cells plated		
	Experiment Number 1	Experiment Number 2	Experiment Number 3
Control	102	95	99
Collagenase for 15 min.	101	100	102
Collagenase for 30 min.	102	96	99

TABLE III

Effect of Different Concentrations of
L-Cell Conditioned Media
on CFUc Growth

Target Cells	Final Concentration (%) of L-Cell Conditoned Media	CFUc (mean colonies/10^5 cells)
Fraction I	1	108
	5	139
	10	154
	15	155
	20	156
Fraction II	1	21
	5	35
	10	62
	15	62
	20	62
Fraction III	1	34
	5	59
	10	61
	15	62
	20	58

TABLE IV

Effect of *in vitro* Misonidazole on CFUc Growth

	Concentration of Misonidazole (uM)	CFUc (mean/10^5 cells plated)
Fraction I (Control)	0	157
	250	150
	500	145
	1000	151
Fraction I (Collagenase)	0	168
	250	166
	500	162
	1000	159
Fraction II	0	34
	250	33
	500	33
	1000	36
Fraction III	0	15
	250	14
	500	14
	1000	12

Effects of *in vivo* Misonidazole on CFUc

The effect of *in vivo* administration of single dose of misonidazole on the growth of bone marrow CFUc is presented in Figure 1. The mean number of colonies per 10^5 cells plated is expressed as a percentage of a control group administered only physiological saline. The mean and standard errors are derived from the pooled results of 10 separate experimental trials. These data suggest that single doses of misonidazole had no cytotoxic effect on the CFUc of fraction I. The same number of colonies were obtained after exposure to misonidazole as were obtained after exposure to equivalent amounts of NaCl. This lack of cytotoxicity for fraction I cells was observed at all doses of misonidazole. Fraction II cells were also relatively resistant to misonidazole's cytotoxic effects. At doses of 0.1 or 0.5 mg/g, similar numbers of CFUc were found in cultures of experimental and control groups. However, a dose of 1 mg/gm of misonidazole resulted in a significant cytotoxicity for the cells of fraction II ($p<.05$), where the surviving fraction of CFUc was reduced to approximately 60% of control values. The cytotoxic effects of misonidazole were the most pronounced with fraction III cells. At all doses of misonidazole which were tested, a significant decrease in the number of surviving CFUc was noted. The growth of CFUc was reduced to between 25% and 40% of control values for this fraction of bone marrow cells. These results are significant at the $p<.001$ level.

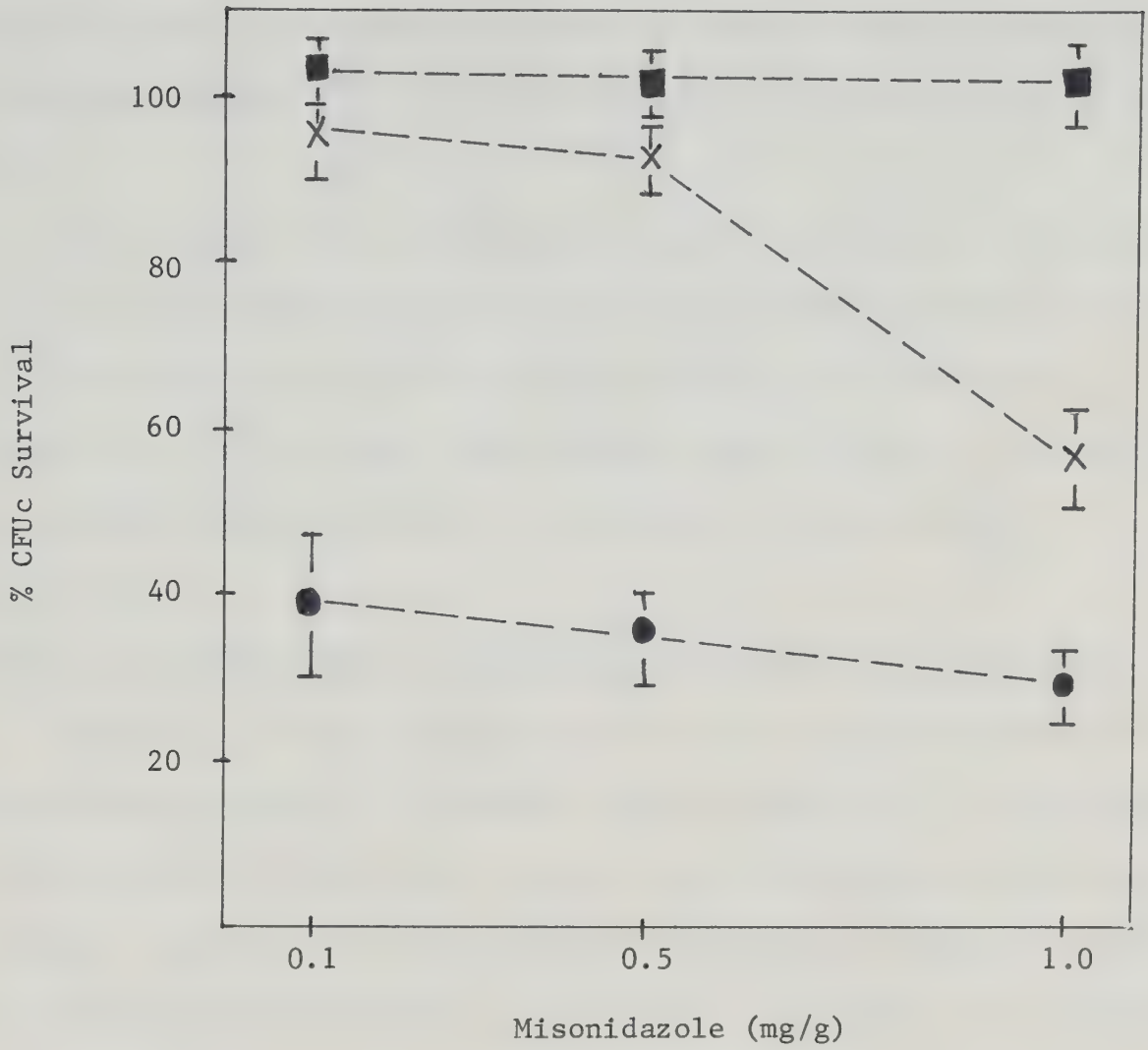

Figure 1. Effect of Misonidazole on CFUc Survival.

Animals received a single dose of misonidazole via intraperitoneal injection. Sixty minutes later, animals were sacrificed and each bone marrow fraction was assayed for surviving CFUc.

■ = fraction I, X= fraction II, ● = fraction III

Effect of *in vivo* Irradiation on CFUc

The effect of *in vivo* administration of a single dose of whole body gamma irradiation is depicted in Figure 2. CFUc from fractions I and II of air breathing demonstrated similar radiosensitivities, with D_o values of approximately 180 rads. The CFUc from fraction III were relatively radioresistant, as compared to those of fraction I and II. This is indicated by the shift in the survival of this fraction curve to the right.

When animals were made hypoxic prior to irradiation, the survival curves of CFUc from fractions I and II were shifted to the right (Figure 3), indicating a resistance of hypoxic cells to the effects of irradiation. In contrast, the survival curve of fraction III CFUc was not significantly altered by the induction of hypoxia prior to irradiation.

Effect of Brief Periods of Anoxia plus Misonidazole on CFUc Growth

Exposure to anoxic conditions for 4 hours did not decrease the proliferative capacity of fraction I CFUc (Figure 4). No difference in CFUc survival was noted when oxic and anoxic cultures were compared. In addition, the presence of misonidazole during oxic incubation of CFUc did not reduce CFUc survival. In contrast, when cells were incubated with misonidazole in anoxic conditons, a significant decrease in survival was noted after one hour for cells exposed to 2 mM misonidazole ($p < .05$). Cells exposed to 1 mM misonidazole exhibited a significantly reduced survival of CFUc after 2 hours ($p < .01$).

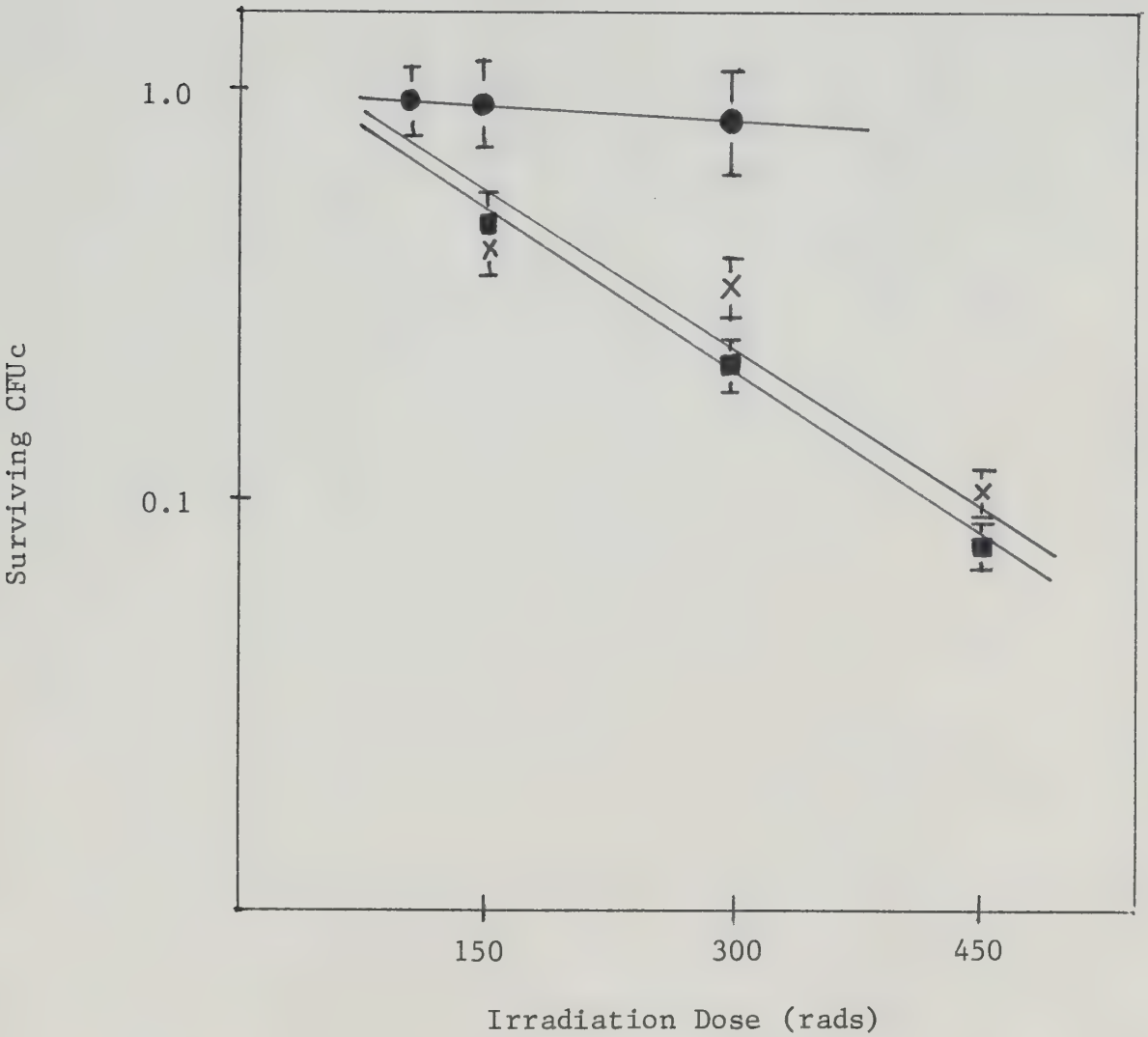

Figure 2. Effect of Whole Body Irradiation on CFUc Survival in Air-Breathing Animals.

Animals were sacrificed immediately following a single dose of irradiation. Each fraction of bone marrow cells was assayed for surviving CFUc.

■ = fraction I, X = fraction II, ● = fraction III

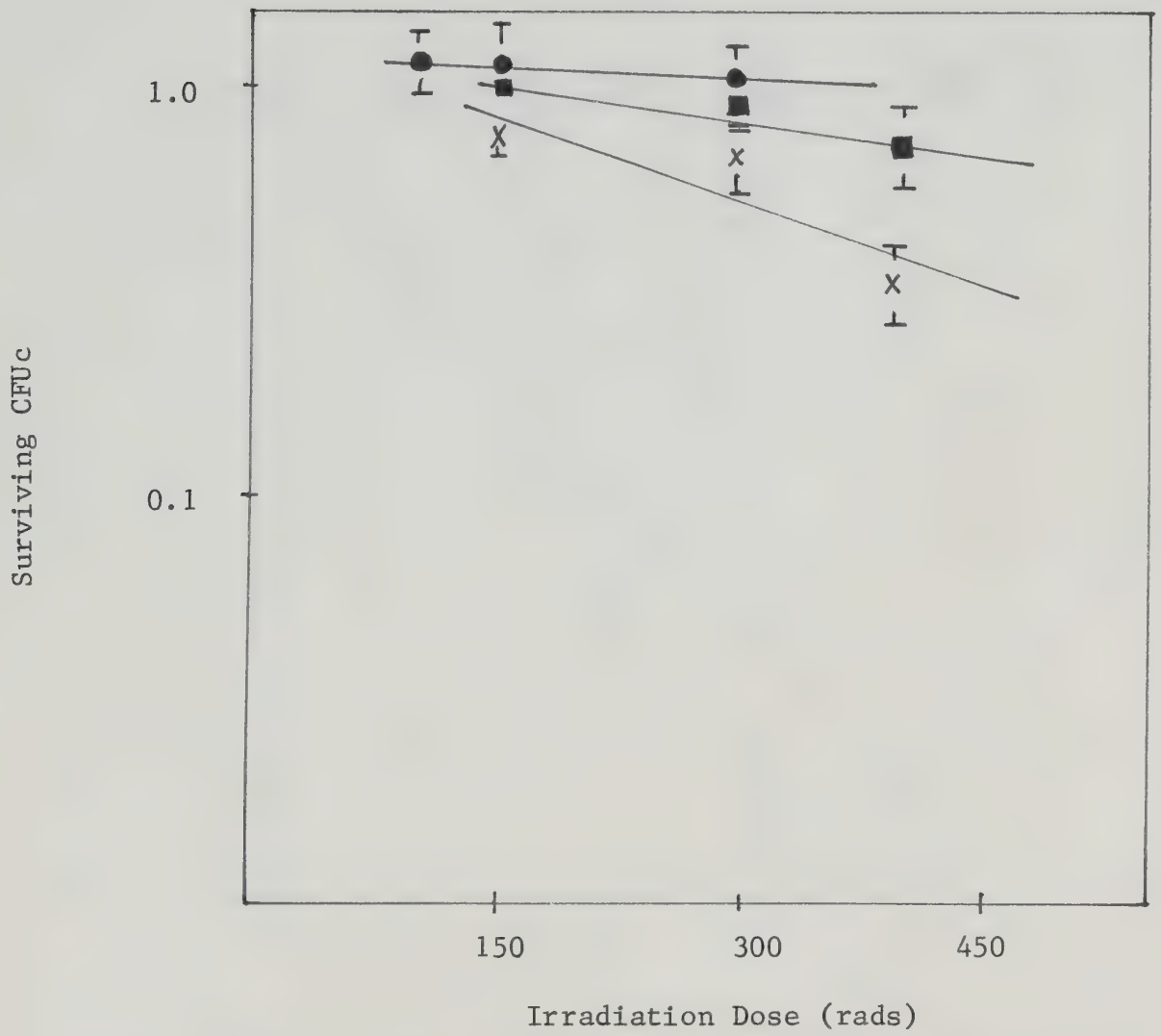

Figure 3. Effect of Whole Body Irradiation on CFUc Survival in Hypoxic Animals.

Animals were made hypoxic by asphyxiation and were given a single dose of irradiation. Each fraction of bone marrow cells was assayed for CFUc immediately following irradiation.

■ = fraction I, X = fraction II, ● = fraction III

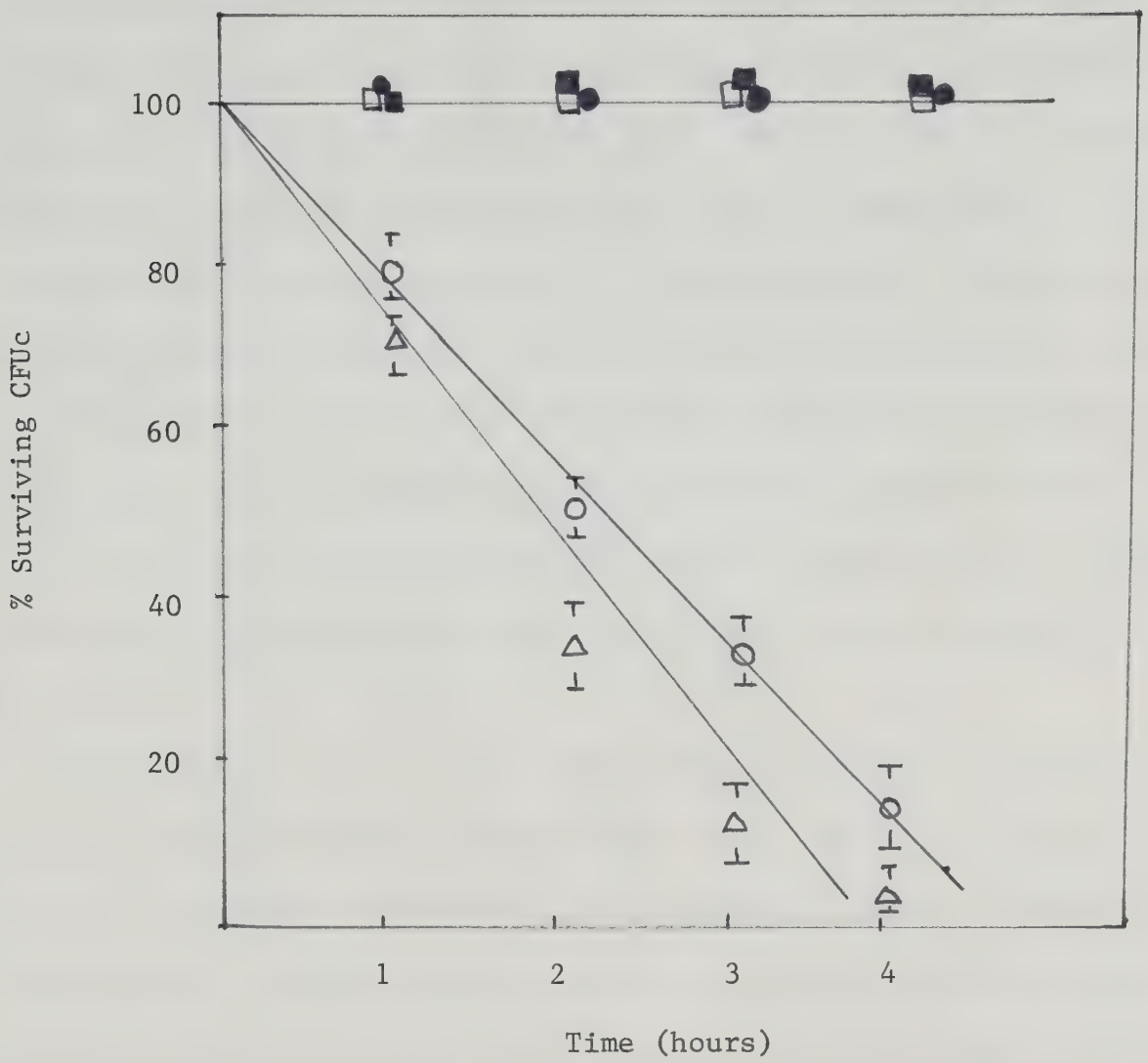

Figure 4. Effects of Brief Periods of Anoxia on CFUc Survival.

Fraction I cells were exposed to air or nitrogen (N_2) with or without misonidazole (MIS) in vitro. Cells were sampled at one hour intervals and assayed for CFUc survival.

■ = air, 1 mM MIS; ● = air, 2 mM MIS;

□ = N_2, no MIS; ○ = N_2, 1 mM MIS; Δ = N_2, 2 mM MIS

Chapter Two

DISCUSSION

Chapter Two describes the results of a series of experiments which have suggested that some cells in the mouse femur exist in a relatively less well oxygenated environment. This conclusion was drawn from studies of the response of bone marrow CFUc to various challenges, such as irradiation and the drug, misonidazole. Because CFUc are exquisitely sensitive to small alterations in their culture conditions such as changes in pH or temperature (40), it was necessary to first establish that the isolation and culture techniques used in this thesis allowed for maximum cell proliferation. If culture conditions were inadequate, differences from the normal response of CFUc may have been masked. Only when culture conditions are optimized can valid conclusions be drawn from comparisons between the growth of control and experimental cultures.

The CFUc assay used in this thesis has been rigorously tested and has consistently provided optimum results for fraction I cells (27, 40). However, two changes in technique were made with regard to fraction II and III cells. First, the cells of fraction II and III were exposed to collagenase during their isolation from the femur, and small amounts of collagenase were transferred to the culture media along with the cells. Therefore, it was necessary to ascertain whether collagenase could interfere with subsequent CFUc growth. Second, the cells of fraction II and III have not been previously characterized with regard to their response to colony stimulating activity. Colony stimulating activity is the single most important component of the culture media, as it serves to promote the growth of CFUc. It was necessary to determine that it

was present in concentrations which were sufficient to allow for maximum cell proliferation. The results shown in Table II and III indicate that the proper culture conditions were attained for these studies.

When testing for possible collagenase cytotoxicity, fraction I cells which were normally never exposed to collagenase during the separation procedure, were used as target cells. These cells were exposed to collagenase for incubation times and at concentrations similar to those used in the separation technique. In addition, the cells were cultured without washing, so collagenase was transferred to the cultures along with the cells, as was usually the case with fraction II and III cells. The results depicted in Table II suggest that collagenase had no cytotoxic effect on CFUc proliferation. The same number of colonies were noted in cultures with or without collagenase. It was therefore assumed that the cells could be separated with collagenase without compromising CFUc growth.

In studies which determined the response of CFUc to colony stimulating activity (CSA), a CSA dilution curve was established for each fraction of bone marrow cells. The growth of bone marrow CFUc in semi-solid cultures is characterized by an initial linear increase in the number of colonies as the concentration of CSA increases. Plateau levels of colony growth are then maintained over a range of CSA concentrations, with colony growth falling off at high concentrations of CSA. This fall off is likely due to the presence of inhibitors at high concentrations of CSA (40). Ideal CFUc culture conditions incorporate concentrations of CSA which are within the plateau range, thereby ensuring the proper balance between optimal stimulation and minimal presence of inhibitors. The data presented in Table III suggests that

the concentration of CSA used in this thesis provided such a proper balance. Similar dilution curves were obtained from each fraction of cells, suggesting that the CFUc from each fraction responded in a similar manner, with each fraction being maximally stimulated with a final concentration of 10% L-cell conditioned media.

These two preliminary experiments ensured that the comparison of control and experimental CFUc culture could be reliably made, as the culture conditions allowed for the optimum proliferation of CFUc.

The studies which first suggested that some stem cells may be in a relatively hypoxic environment utilized the selective hypoxic cell cytotoxic effect of the drug misonidazole (13-17). The data depicted in Figure 1 summarize a series of experiments which investigated the effects of in vivo administration of a single dose of misonidazole on bone marrow stem cells. In these experiments, misonidazole was used at relatively low doses. In mice, high doses of misonidazole have been associated with a lowering of body temperature, heart rate and respiration (46,47). By selecting doses well below the acute toxic dose (range of LD50/3=1.4-1.8 mg/g) (46, 47), it was possible to investigate misonidazole's cytotoxicity for hematopoietic cells without the complications of whole body side-effects. The results shown in Figure 1 confirm that small doses of misonidazole are cytotoxic for some bone marrow cells. The CFUc from fractions I and II were relatively unaffected by misonidazole, with a drop in CFUc being noted in fraction II with the use of 1 mg/g misonidazole. This decrease in CFUc may reflect a senstivity to the cytotoxic effects of the drug which is only expressed at relatively large doses. Hall and Roizin-Towle (17) have described a misonidazole cytotoxicity for aerobic cells which is

associated with high doses of misonidazole. Because the CFUc from fraction II did not decrease after smaller doses of misonidazole, it is likely that the drop in CFUc associated with a dose of 1 mg/g reflects a high dose toxicity.

The data obtained from fraction I cells, and from fraction II cells with smaller doses of misonidazole is in agreement with other published reports of the effect of misonidazole on murine stem cells. Turner et al. could not demonstrate any misonidazole-associated cytotoxicity in normal or tumor bearing animals (18). These observations were based on standard CFUc and CFUs assays using fraction I cells and comparable doses of misonidazole. In other studies, Pettersen (48) investigated the effects of misonidazole on the proliferation of CFUs. Using fraction I cells and doses of misonidazole which were comparable to those used in this thesis, Pettersen concluded that misonidazole was not cytotoxic for pluripotential stem cells. The similar results obtained in this thesis for fraction I and II cells have prompted the same conclusion. That is, misonidazole was not cytotoxic for the CFUc found in the medullary cavity or near the endosteum.

This observation cannot be extended to all of the stem cells in the mouse marrow. The CFUc which were isolated from the compact bone clearly demonstrated a cytotoxic response to _in vivo_ exposure to misonidazole. A significant decrease in CFUc was noted for all doses of misonidazole tested with increasing cytotoxicity associated with increasing doses of misonidazole. Only 25% to 45% of the CFUc from fraction III were able to survive _in vivo_ challenge with misonidazole at doses which ranged from 0.1 to 1.0 mg/g (one hour exposure). This cytotoxicity seen with fraction III cells is similar to that described

by Allalunis et al. (19) in a study of human cancer patients receiving misonidazole. In this clinical study, a decrease in bone marrow CFUc was noted after ingestion of misonidazole.

The cytotoxicity noted in these studies was clearly an *in vivo* event. A decrease in bone marrow CFUc could not be demonstrated by exposing aerobic cells to misonidazole *in vitro*. The data presented in Table IV illustrate several important points. First, *in vitro* exposure to concentrations of misonidazole in excess of those attainable *in vivo* (ie. 1000 mM), did not result in any significant cytotoxicity for CFUc. Second, no cytotoxicity was observed when the time of exposure to misonidazole was significantly increased. Misonidazole was present in the CFUc assay for seven days. In contrast, after *in vivo* administration, most of the misonidazole was excreted within four hours (49). This increased exposure time did not produce any cytotoxicity. Third, the presence of collagenase did not alter the action of misonidazole on CFUc *in vitro*. This was observed when fraction I cells, assayed with and without exposure to collagenase, expressed no difference in the number of colonies at the end of the culture period. Lastly, misonidazole did not demonstrate any selective cytotoxicity for any particular fraction of bone marrow cells exposed *in vitro*. This suggests that fraction III cells are not inherently sensitive to misonidazole. In contrast, *in vivo* exposure to misonidazole resulted in a significant toxicity for fraction III cells.

These results have suggested that *in vivo*, fraction III cells may reside in a relatively hypoxic microenvironment. The cytoxicity of misonidazole for hypoxic cells has been well documented (11-17). In both *in vivo* and *in vitro* experiments, the presence of relatively

hypoxic conditions are a prerequisite for the demonstration of misonidazole cytotoxicity. Only when large doses of misonidazole are used, can a cytotoxicity be demonstrated for aerobic cells (17). Therefore, the decrease in CFUc after a small *in vivo* dose of misonidazole suggests that those cells could be relatively hypoxic. When fraction III cells were removed from their microenvironment and maintained in aerobic culture, misonidazole cytotoxicity could not be demonstrated, suggesting that the microenvironment of those cells is relatively less well-oxygenated. If the cells themselves were inherently sensitive to misonidazole, it would be expected that such a sensitivity could be demonstrated *in vitro* as well. This was not the case.

The exact mechanism which results in the cytotoxicity seen with misonidazole is not clear. Varghese and co-workers have postulated that the binding of the nitro-reduction products of misonidazole to macromolecules within the cells is responsible for misonidazole's cytotoxic effect (50,51). The formation of these reduction products has been closely associated with the metabolism of misonidazole by hypoxic cells, and is thought to be responsible for the preferential cytotoxicity of misonidazole for hypoxic tissues (50, 51). Rowley and co-workers have extended these observations to the study of the effect of nitro-reduction products on DNA (52). They have determined that the major effects of the reduction products are to induce cleavage of the DNA molecule at adenine or thymine bases, and have suggested that this type of damage is similar to that induced by bleomycin. It is also possible that other vital cellular structures, such as the membrane, could be a target of misonidazole's damage. However, the exact nature

of misonidazole's cytotoxicity remains unclear. Studies of the mechanism of misonidazole's cytotoxicity have yet to be extended to hematopoietic stem cells.

The theory that stem cells in the compact bone are relatively hypoxic is also supported by studies of the response of these CFUc to ionizing irradiation. When aerated cells are irradiated, molecular oxygen can fix the free radicals which are generated during irradiation, and prevent the repair of damaged macromolecular structures (54). In the absence of oxygen, many of the radiation-induced radicals can be repaired by rapid chemical reactions within the cell.

This oxygen effect has been demonstrated for murine stem cells. In separate studies, Till (55), Phillips et al (56, 57) and Turner et al. (18) have compared the surviving fractions of CFUs which were obtained from animals irradiated in either hypoxic or air-breathing states. The results demonstrated that the CFUs from animals which were hypoxic at the time of irradiation were relatively resistant to the effects of irradiation. This resistance was expressed as an increased survival of CFUs relative to the survival of CFUs from air breathing animals. This demonstration of an oxygen effect has been taken as evidence that the bone marrow is a well-oxygenated tissue.

Studies in this thesis confirm these results, but suggest that this observation of an oxygen effect can only be extended to the bone marrow cells in the medullary cavity and near the endosteum. CFUc found in the compact bone were relatively resistant to the effects of irradiation. The experiments described in Figure 2 show that the CFUc from fractions I and II were sensitive to the effects of irradiation, with an exponential decrease in survival with increasing radiation dose. The D_o

values determined for these two fractions are similar to those which have been reported by other investigators (58-59). The radiation sensitivity of fraction I and II can be altered by making the animals hypoxic prior to irradiation. This alteration is expressed by a shift in the survival curves to the right, a phenomenon identical to the oxygen effect previously described.

In contrast the CFUc from fraction III appeared to be less sensitive to irradiation. More than 80% of these cells survived irradiation doses of 150 or 300 rads, which were lethal for more than 60% of the CFUc from fractions I and II. In addition, a significant oxygen effect could not be demonstrated for this fraction. That is, no change in the radiation sensitivity of CFUc from fraction III was observed for mice killed minutes prior to irradiation. The post irradiation survival of CFUc from hypoxic animals was similar to that observed from oxic animals. Because most mamalian cells have been shown to have oxygen enhancement ratios of approximately 2-3 (56, 57), these results suggest that the CFUc of the compact bone may be in a relatively hypoxic microenvironment. If these cells were well-oxygenated, an oxygen enhancement should have been demonstrated, as was the case with fraction I and II CFUc. This suggestion of a relatively hypoxic microenvironment complements the evidence of hypoxic state of fraction III from the misonidazole cytotoxic studies.

Other hematopoietic elements have been shown to be relatively radioresistant as compared to the medullary CFUc or CFUs. D_o values of 200 to 444 rads have been attributed to bone marrow stromal cells (60-63). These stromal cells are an integral part of the hematopoietic microenvironment. In addition to supporting hematopoiesis <u>in situ</u>, the

transfer of stromal elements to heterotopic sites can result in the regeneration of hematopoiesis. The relationship of stromal cell to other hematopoietic elements is not clear. But the demonstration of other radioresistant elements in the marrow suggests that radiation response of the tissue is not as uniform as was once assumed, and gives credence to the possibility that sub-populations of hematopoietic cells may express different radiation sensitivities.

Experiments studying the effects of hypoxia on CFUc also confirmed that some stem cells may reside in a relatively less well oxygenated microenvironment. In these studies, fraction I cells were incubated with or without misonidazole under aerated or hypoxic conditions. Fraction I cells were chosen as the target because experiments with both misonidazole and irradiation have suggested that this population of cells is relatively well oxygenated _in vivo_. It was therefore expected that if CFUc survival was incompatible with hypoxic conditions, any compromise in CFUc growth would be most apparent with this group of cells.

Two conclusions were drawn from the experiments which used nitrogen chambers which are summarized in Figure 3. First, the data show that fraction I cells could survive brief periods of hypoxia with virtually no decrease in survival. These results are similar to those described by Bradley et al. (64). In assays of both human and murine CFUc, Bradley et al. demonstrated that incubation of cultures at reduced oxygen tensions resulted in increased colony formation and increased colony size. This observation of improved colony growth at lowered oxygen tensions has prompted other investigators to suggest that reduced oxygen tension in the marrow may serve as a controlling mechanism in the

hematopoietic process (65, 66). By demonstrating that CFUc can survive in the complete absence of oxygen, the results described in this thesis add additional support for the theory that *in vivo*, differences in marrow oxygenation can regulate the hematopoietic process.

In addition to demonstrating that stem cells could survive in hypoxia, the results from the nitrogen chamber experiments provided data which paralleled that obtained from the studies of misonidazole's effect on CFUc, which were described in the first part of this chapter. The undiminished CFUc survival seen after the aerated incubation of fraction I cells with misonidazole *in vitro* again suggested that fraction I cells are likely to be well oxygenated *in vivo*. Of interest is the rapid and significant decline in CFUc survival which was noted for cells incubated with misonidazole under hypoxic conditions. A decrease in CFUc survival was noted after 60 minutes of incubation, with progressive decreases in survival being noted over the course of the experiment. This decline in CFUc survival with time expands the observation of Stratford who noted that both drug concentration and time of exposure are important features of misonidazole's cytotoxicity (67). Furthermore, the decrease in CFUc survival noted after exposure to misonidazole under hypoxic conditions *in vitro* was similar to that observed in fraction III cells exposed to misonidazole *in vivo*. Such a similarity provided indirect evidence to suggest that fraction III cells may be relatively hypoxic.

Some anatomical studies of the circulation of the bone and bone marrow have addressed the question of bone marrow oxygenation. In a study of felines, Misrahy et al. demonstrated that the bone cortex has a reduced pO_2 relative to that of the marrow (68). Brooke, using rodent data, also described a reduced pO_2 in the compact bone, along with an

elevated pCO_2 and a decreased circulation rate (69, 70). Both of these works suggest that the compact bone is oxygenated, but to a lesser extent than the medullary cavity. The relatively reduced oxygen tension described by Brooks and Misrahy et al. may be sufficient to account for the response of CFUc from fraction III to misonidazole and irradiation. Stratford has shown that misonidazole's cytotoxicity is a function of oxygen tension, and that complete anoxia is not required to demonstrate misonidazole-associated toxicity (71). For example, nerve fibers have been shown to be sensitive to misonidazole's cytotoxic effects. This sensitivity results in the clinical dose-limiting neurotoxicity (72). In this case, it is the relatively reduced oxygenation of neural tissue which is thought to be responsible for misonidazole associated damage. In an analagous manner, the reduced oxygen tension of some bone marrow cells may render them sensitive to misonidazole's cytotoxic properties. A reduced oxygen tension would also explain the response of fraction III CFUc to irradiation, as relative hypoxia has been shown to decrease the sensitivity of cells to the effects of ionizing irradiation (26).

CHAPTER THREE

Possible Role of a Hypoxic Sub-Population of Stem Cells in Marrow Regeneration

Chapter Three

RESULTS

Animal Survival After Irradiation

Pre-treatment with multiple doses of misonidazole reduced the ability of animals to survive whole body irradiation. Combined results of two experimental trials are depicted in Figure 5. When saline and misonidazole treated animals were compared, a difference in the LD 50/30 dose of irradiation was noted [LD 50/30 (saline) = 586 rads; LD 50/30 (Misonidazole) = 450 rads]. This misonidazole enhancement of irradiation lethality equals a dose modifying factor (DMF) of 1.3. All of the animal deaths observed in these studies occurred 7 or more days following treatment.

Animal Survival After Cyclophosphamide

Pre-treatment of animals with misonidazole reduced their ability to survive challenge with cyclophosphamide. Combined results of four experimental trials is presented in Figure 6. Misonidazole pre-treatment reduce LD 50/30 dose of cyclophosphamide to 340 mg/kg. The LD 50/30 dose of cyclophosphamide was 410 mg/kg for animals which were pre-treated with saline. (DMF = 1.2). The majority of the deaths observed in these animals occurred 7 or more days after treatment with cyclophosphamide.

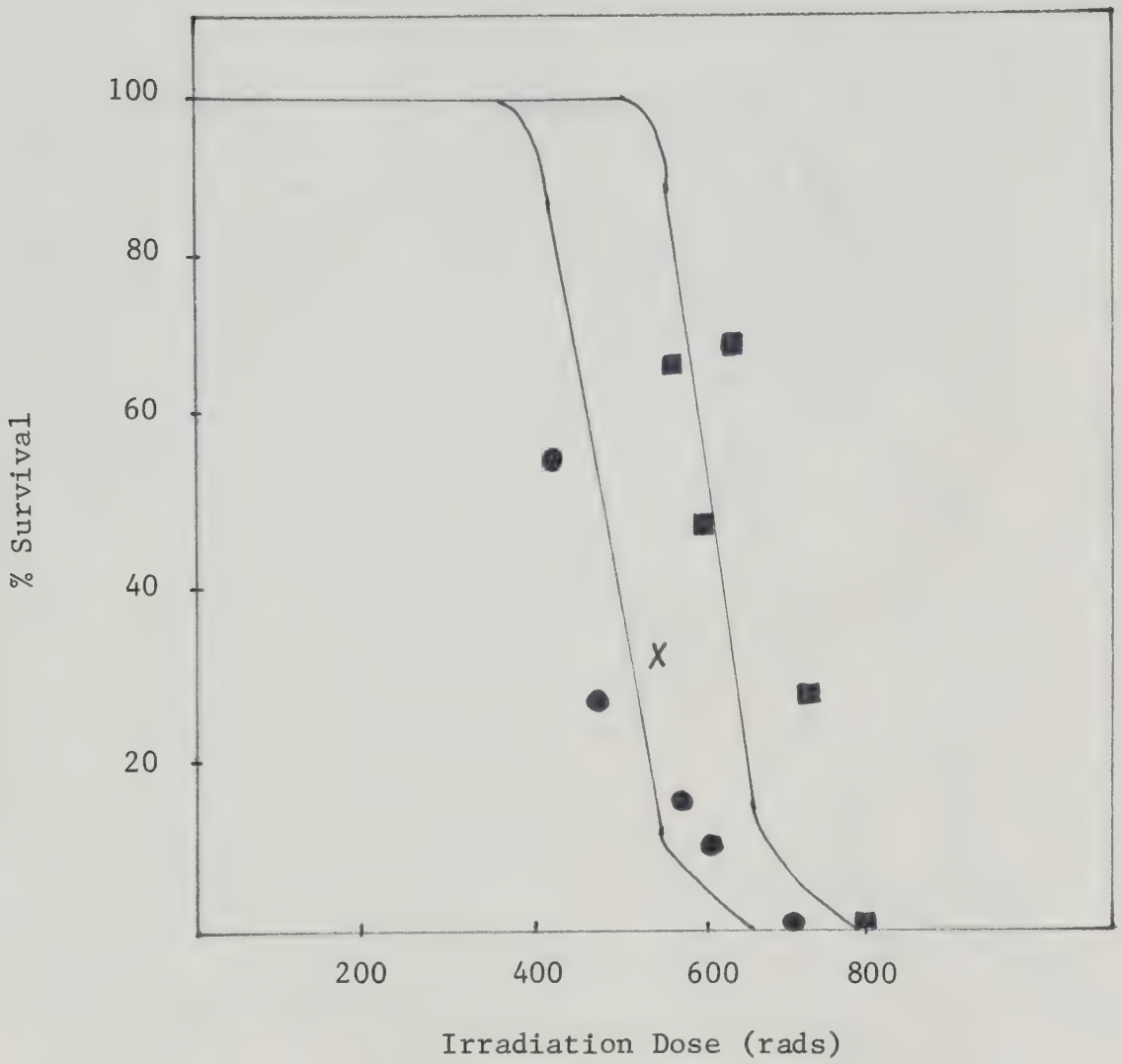

Figure 5. Misonidazole Enhancement of Irradiation Induced Lethality.

Animals were pre-treated with misonidazole (0.2 mg/g at 0 minutes, 0.1 mg/g at 40, 120 and 180 minutes). At 210 minutes, animals received a single dose of whole body irradiation. Animal survival was monitored over 30 days.

● = misonidazole, ■ = control, X = 0.5 mg/g misonidazole after irradiation

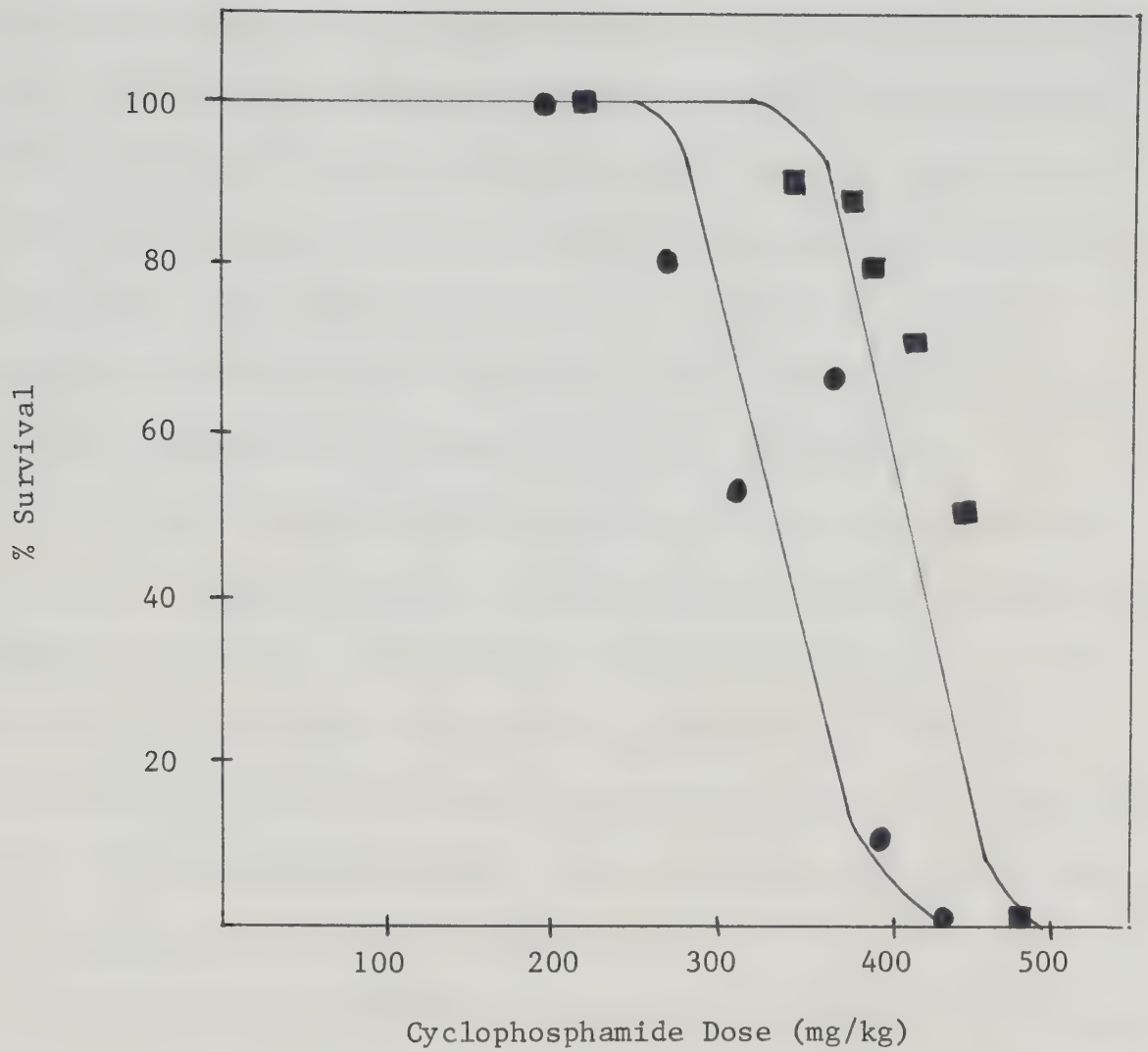

Figure 6. Misonidazole Enhancement of Cyclophosphamide Induced Lethality.

Animals were pre-treated with misonidazole (0.2 mg/g at 0 minutes, 0.1 mg/g at 60, 120 and 180 minutes). At 210 minutes, animals received a single dose of cyclophosphamide. Animals survival was monitored for 30 days.

● = Misonidazole, ■ = Control

Chapter Three

DISCUSSION

The contribution made by each sub-population of cells to the hematopoietic process has yet to be determined. However, the work of several investigators has suggested that the cells in the compact bone may be instrumental in the re-establishment of hematopoiesis following insult to the marrow. Maloney and Patt were the first to show that the cells resident in the bone were responsible for the regeneration of active hematopoiesis in a femur which has been mechanically depleted of cells (73). This observation was later extended to include the depletion of the femur by irradiation. The same conclusion regarding the local origin of regenerating tissue was reached (74).

The cells referred to by Maloney and Patt may be related to fraction III cells described in this thesis, since both populations are resident in the bone. If fraction III cells do contribute to the process of hematopoietic regeneration, then the combined use of misonidazole and known myelotoxic agents could result in a compromised marrow recovery, as fraction III cells have been shown to be sensitive to the cytotoxic effects of misonidazole.

Misonidazole is currently undergoing clinical trials to determine its effectiveness as a hypoxic cell sensitizing agent when used with irradiation (75, 76) or chemotherapy (77). Although the architecture of the human and the murine marrow differs, it has been demonstrated that the human bone marrow contains a population of cells which can re-establish active hematopoiesis following injury to the marrow (78, 79). Therefore, studies with murine marrow of the effects of combining misonidazole with irradiation of chemotherapy may provide some

preliminary indication as to whether enhanced myelotoxicity may be expected with this combination of agents in the clinical setting.

In the studies described in this thesis, myelotoxicity was assessed by means of comparative animal survival. When animals are given irradiation or chemotherapy, those which die between three and five days following treatment are thought to have succumed to gastrointestinal failure (78). Deaths which are noted after this time are usually ascribed to hematopoietic failure (78, 79).

The survival studies in this thesis compared animals treated with misonidazole or physiological saline before challenge with irradiation or cyclophosphamide. Careful note was made of the time of death of all the animals. Figure 4 depicts the results of misonidazole pre-treatment on the survival of animals following a single dose of irradiation. A reduction of the LD 50/30 dose of irradiation was noted for the animals which had been pre-treated with misonidazole. This decreased survival associated with misonidazole treatment suggested that misonidazole enhanced the lethality associated with irradiation.

This enhancement could have occurred in one of two ways. Misonidazole has both radiosensitizing and cytotoxic effects on hypoxic cells. In studies designed to distinguish between two properties, a group of animals received multiple doses of misonidazole after irradiation. If the enhanced lethality associated with misonidazole use was due primarily to its radiosensitizing effects, then the animals which were treated with misonidazole after the irradiation was completed should have had the same survival rate as those animals which received saline before irradiation, since when it is given after irradiation, misonidazole can no longer act as a radiosensitizer. On the other hand,

if the cytotoxicity associated with misonidazole was largely responsible for the observed increase in lethality, then the administration of misonidazole after irradiation would be expected to result in the same survival rate observed in animals treated with misonidazole before irradiation. As is shown in Figure 4, the observed enhancement of irradiation lethality was likely due to misonidazole's cytotoxic effects. Animals that received misonidazole either before or after irradiation had a reduced survival rate (23% and 30% survival at 530 rads) as compared to the survival rate of animals that did not receive misonidazole (50% survival at 530). Most of the deaths in these experiments occurred after five days, suggesting bone marrow failure as the likely cause of death.

Fraction III cells may have been the target of this observed cytotoxicity, as experiments described earlier in this thesis have demonstrated that these cells are sensitive in vivo to misonidazole's cytotoxic effects. Another group of animals was incorporated in these irradiation studies to permit a comparison of the results obtained in the CFUc assays and irradiation experiments. These animals received a single, large dose of misonidazole before irradiation. This single dose group had a survival rate similar to that of animals treated with multiple doses of misonidazole. This finding indicated that an extended exposure of misonidazole was not necessary to demonstrate its cytotoxic effect, and suggested that a single dose of misonidazole, which reduced CFUc survival also reduced animal survival.

Although studies demonstrating that treatment with misonidazole after irradiation reduced animal survival suggested that misonidazole's cytotoxic effects were largely responsible for the enhanced lethality,

the possibility that misonidazole may radiosensitize some bone marrow cells cannot be completely excluded. In studies of the effects of chemotherapeutic agents and irradiation on normal tissues, Phillips and Fu postulated that agents that bind to DNA are capable of sensitizing normal tissues to irradiation (80). The binding of the nitro-reduction products of misonidazole has been offered as a mechanism to explain its cytotoxic effects in the absence of irradiation (51). If the observation of Phillips and Fu can be extended to these studies, then the binding of misonidazole's nitro-reduction products may also serve to sensitize some less well oxygenated bone marrow cells to the effects of irradiation, thereby contributing to the decreased survival associated with misonidazole therapy. Although such a radiosensitizing effect may have occured it could not have accounted for all of the decreased survival noted in these experiments, as treatment with misonidazole after irradiation resulted in a similar survival rate as treatment before irradiation.

The findings that animal survival after irradiation was reduced when animals were treated with misonidazole is the first observation of this phenomenon (81). This observation may have important clinical significance, particularly for half body radiotherapy as previous clinical and experimental work with misonidazole has been based on the premise that misonidazole does not enhance damage to normal tissues when used with irradiation (82, 83). The bone marrow has been assumed to be a uniformly well oxygenated tissue which would be spared the sensitizing effects of misonidazole. Work described in this thesis has demonstrated that this is not the case, and suggests that a re-evaluation of earlier studies of the effects of misonidazole on hematopoietic tissue may be of value.

Other experiments described the effects of misonidazole on the survival of animals which had received cyclophosphamide. These findings paralleled those observed in the irradiation survival studies, that is, pretreatment with misonidazole resulted in decreased animal survival. Misonidazole treatment reduced the LD 50/30 dose of cyclophosphamide from 410 mg/kg to 340 mg/kg (Figure 6). Most of the animal deaths occurred at day 8, or later, suggesting that failure of the bone marrow to recover from cyclophosphamide-induced damage was the likely cause of death. The cytotoxic effects of cyclophosphamide on the bone marrow are well documented (83-86). The sensitivity of fraction III cells to misonidazole's cytotoxic effects has also been established. This observed increase in mortality seen in mice treated with both misonidazole and cyclophosphamide was similar to an additive toxicity which would be expected from the combination of two cytotoxic agents.

Other investigators have described a similar increase in toxicity when misonidazole and chemotherapeutic agents were combined. Using in vitro tumor cell assays, Stratford et al. demonstrated that preincubation of hypoxic cells with misonidazole resulted in increased toxic effects of melphalan, mustine hydrochloride and Cis-dichloroammineplatinum (II) (84). Using a Lewis lung tumor system, Rose et al. demonstrated enhanced tumor cell kill when misonidazole was combined with melphalan (85). The studies by Rose et al. also described an enhanced myelotoxicity when misonidazole and melphalan were combined, but the authors suggested that such myelotoxicity may be acceptable in light of the enhanced therapeutic gain afforded the tumor. Clement et al. reached similar conclusions in their study of misonidazole enhancement of the cytotoxicity of various alkylating agents (86).

These workers concluded that misonidazole in combination with alkylating agents was not toxic for the marrow. They suggested that the reduced CFUs survival, noted after the administration of misonidazole alone, and the reduction in the LD10 dose of cyclophosphamide administered after misonidazole, did not represent significant toxicities, and were likely due to experimental error.

The results in this thesis do not support the conclusions drawn by Rose et al. and Clement et al. These workers have suggested that the bone marrow is at little risk when misonidazole is combined with chemotherapy. The enhanced myelotoxicity seen with fraction III cells exposed to misonidazole argues against the suggestion that the marrow is not affected by misonidazole. In addition, the decreased survival of animals treated with misonidazole before irradiation or cyclophosphamide suggests that damage to this normal tissue could possibly offset any potential therapeutic gain. In related work, Pedersen et al. could not demonstrate a therapeutic gain when both misonidazole and cyclophosphamide were administered to mice bearing the Lewis lung tumor (87). In these studies, a significant reduction in survival was noted in animals which were treated with both agents, as compared to those treated with cyclophosphamide alone. This observed damage to normal tissue prompted the authors to conclude that although the combined use of misonidazole and cyclophosphamide resulted in a modest enhancement of tumor kill, the accompanying damage to normal tissue limited any therapeutic benefit to be derived from such a combination of agents.

Other studies by Allalunis et al. demonstrated that misonidazole pretreatment reduced survival and delayed recovery of bone marrow CFUc after relatively small doses of cyclophosphamide (88), suggesting that misonidazole enhances damage to the bone marrow when used at clinically relevant concentrations.

General Discussion and Summary

In conclusion, these studies have suggested that a sub-population of hematopoietic stem cells may be hypoxic, and that these cells may play a role in the regeneration of hematopoiesis after injury to the marrow.

The findings described in this thesis are consistent with those of other investigators who noted the presence of significant numbers of stem cells in areas outside the medullary cavity. The partial characterization of the cells found in different microenvironments expanded earlier reports which were limited to the quantitation of these cells. In addition to describing a method for improved cell recovery, this thesis details the numbers and types of cells found in different microenvironments, and compares the effects of age, gender and genetic strain on these cell populations.

Also included was a description of some features of these cells in tissue culture assays for CFUc. Notably, it was determined that while the cells from each microenvironment responded to colony stimulating activity in the same manner, the CFUc from the compact bone consistently produced smaller, more compact colonies.

The studies in this thesis have also challenged the assumption that the bone marrow is a uniformly well oxygenated tissue. Hematopoietic stem cells from different microenvironments were found to respond differently to the hypoxic cell sensitizer, misonidazole, and to irradiation. Specifically, the cells in the compact bone were sensitive to the cytotoxic effects of misonidazole, and were relatively resistant to the effects of irradiation. Both of these responses, common to cells which are hypoxic, suggests that the cells in the bone may reside in an environment of reduced oxygen tension.

Control studies which demonstrated that CFUc could survive brief periods of anoxia without significant loss of proliferative capacity supported the contention that cells in the bone may be relatively hypoxic and retain viability. This theory was also supported by the work of other investigators who measured the pO_2 and blood circulation of the compact bone and demonstrated that bone is characterized by a reduced oxygen tension and a reduced blood flow.

The results which demonstrated that some CFUc are sensitive to the cytotoxic effects of misonidazole were similar to those obtained from a study of cancer patients who participated in a clinical trial of misonidazole. In the human studies, a decrease in each patient's bone marrow CFUc was noted after ingestion of misonidazole. A similar decrease in the CFUc from the compact bone was noted in animals that were injected with misonidazole. The work in this thesis is the first report of a misonidazole cytotoxicity of murine hematopoietic stem cells. Previous studies using unseparated marrow had failed to detect any significant sensitivity to misonidazole.

The studies in this thesis also expanded the suggestion of other investigators that the cells in the bone may contribute to the process of hematopoietic regeneration. By using the sensitivity of fraction III cells to misonidazole as a tool, it could be determined that when the cells in the compact bone were partially depleted, animals had an impaired ability to recover from a challenge with cyclophosphamide or irradiation. This impairment was reflected in a reduced survival for animals that were treated with both misonidazole and another myelotoxic agent.

The misonidazole enhancement of irradiation lethality described in this thesis is the first report of this phenomenon. Previous *in vivo* studies which combined misonidazole with irradiation focused on the effects of such treatment on tumor burden, and have largely neglected the possibility of significant damage to normal tissues.

The enhanced cytotoxicity associated with the combined use of misonidazole and chemotherapy has been previously described. However, other investigators have suggested that the enhanced myelotoxicity which was observed in some studies was not significant. The demonstration in this thesis that the combined use of misonidazole and cyclophosphamide reduced the ability of the bone marrow to recover from injury prompts the conclusion that the enhanced tumor cell kill afforded by such treatment may be accompanied by significant, dose-limiting damage to the marrow.

When viewed as a whole, the studies described in this thesis have suggested that it may no longer be appropriate to regard the hematopoietic elements of the bone marrow as a homogeneous tissue. Differences in the degree of oxygenation, response to misonidazole and irradiation, and recovery from challenge with myelotoxic agents suggests that a model which separately evaluates the stem cells from each microenvironment may be more appropriate. The results described in this thesis have provided the groundwork for future experimentation using such a model.

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